Nematicidal activity of volatile fatty acids generated from wheat bran in reductive soil disinfestation

Article *in* Japanese Journal of Nematology · January 2009

DOI: 10.3725/jjn.39.53

CITATIONS
26

READS
86

6 authors, including:

Takayuki Mizukubo
Maruwa Biochemical Co., Ltd.
81 PUBLICATIONS **586** CITATIONS

SEE PROFILE

Nematicidal activity of volatile fatty acids generated from wheat bran in reductive soil disinfestation[1]

Masahiko Katase[2], Chikako Kubo[2,3], Shingo Ushio[2], Eiichi Ootsuka[2], Taeko Takeuchi[2] and Takayuki Mizukubo[4]

We tried to elucidate the mechanisms of soil disinfestation by a combined treatment of wheat bran amendment, temporary flooding, and tarping with plastic film (reductive soil disinfestation, RSD). In a laboratory experiment, redox potential decreased to under -200 mV in RSD-treated soil and short-chain volatile fatty acids (VFAs), such as acetic and n-butyric acids, were generated at levels of 9.8 to 10.8 mM in the soil solution in 48 hr. VFAs showed high nematicidal activities when second-stage juveniles of *Meloidogyne incognita* were exposed to them at 10 mM for 24 hr. Acetic and n-butyric acids had nearly equal nematicidal activity and their mixtures showed an additive effect. VFAs increased the nematicidal activity with decreasing pH, indicating that nematicidal activities of VFAs are related to their ionization. Logistic LC_{50} of nonionized acetic acid was estimated to be 5.6 $\pm$ 0.2 (SEM) mM. In a field demonstration, RSD was confirmed to be adequate for nematode control, and 5.7 mM of acetic acid and 1.5 mM of n-butyric acid were calculated in the water-phase of the soil, although the soil pH was not low enough to form nonionized acids at the lethal concentration. These results show that VFAs generated by soil microorganisms in reduced soil probably play an important role in nematode suppression in practice. Nematol. Res. 39 (2), 53-62 (2009).
Key words : cultural control, organic amendment, root-knot nematode, soil microorganism, soil reduction.

INTRODUCTION

For the cultural control of plant-parasitic nematodes, soil amendment with fresh or composted organic matter, such as animal wastes, crop residues, manure, green manure, sugarcane bagasse, bone meal, corn meal, and compost, has long been studied (Norton, 1978; Nakasono, 1989; Sikora, 1992). Especially, amendment with labile organic matter was found to show quick nematicidal activity. The mode of action, however, has not yet fully been revealed. Short-chain volatile fatty acids (VFAs) contained in organic matter or produced through the decomposition of the organic matter were pointed out as a factor of the action mechanism (Sayre *et al*., 1965; Hollis and Rodriguez-Kabana, 1966; Lynch, 1978; Badra et al., 1979; McBride *et al*., 2000).

Nematode control with soil amendment has been improved by combining it with flooding and solarization (Hollis and Rodriguez-Kabana, 1966; Sotomayor *et al*., 1999). Shinmura *et al.* (1999) developed a simple and low-cost soil disinfestation method for Fusarium root rot of Welsh onion in a greenhouse. The method consisted of wheat or rice bran applied at 1 to 2 t/10a and temporary flooding to bring soil moisture up to field capacity followed by tarping with transparent polyethylene film to keep a high soil temperature for about 20 days. This method, reductive soil disinfestation (RSD), is believed to reduce redox potential of soil under anaerobic conditions to exhibit its control effect. RSD is recognized to be effective for controlling plant-parasitic nematodes, such as *Meloidogyne* spp. and *Pratylenchus* spp., as well as soil-borne fungal pathogens, such as *Pyrenochaeta* spp., *Fusarium* spp. and *Phomopsis* spp. (Kubo et al., 2004; Katase *et al*., 2005; Kubo and Katase, 2007). Hence, RSD has been widely used in Japan for greenhouse cultivation of tomato, cucumber, watermelon, melon, strawberry, kidney beans, peas, and spinach (Kubo and Katase, 2007).

Reductive soil disinfestation requires soil temperature of about 30°C, which is lower than the soil temperature required for soil solarization. Therefore, RSD can be practiced over a wide range of seasons and regions. In addition, the high soil temperature for RSD needs to be maintained for about 10 days during a hot summer and about 20 days in warm seasons; the required durations are shorter than that for soil solarization. Wheat bran is massively produced as a by-product in flour milling processes and partly distributed as a commercial feed for livestock. It is uniform in

[1] A part of this paper was presented in the annual meeting of the Japanese Nematological Society in 2001, 2002, 2003, 2004, and 2005.

[2] Chiba Prefectural Agriculture and Forestry Research Center, Daizenno-cho 808, Midori-ku, Chiba 266-0006, Japan.

[3] Present address: Chiba Prefectural Chiba Agriculture and Forestry Promotion Center, Ohganezawa-cho 473-2, Midori-ku, Chiba 266-0014, Japan.

[4] National Agricultural Research Center, Kan-nondai 3-1-1, Tsukuba, Ibaraki 305-8666, Japan.

quality, cheap and easy to obtain. For these reasons, RSD has been used as an effective and feasible technique for controlling nematodes and soil-borne diseases in sustainable agriculture.

We performed laboratory experiments and a field demonstration to elucidate the mechanism of RSD on the basis of VFAs generation and their nematicidal activities.

MATERIALS AND METHODS

Measurement of dissolved oxygen and redox potential in a laboratory experiment:

In order to estimate anaerobic conditions and reduced status of soil, dissolved oxygen (DO) and redox potential (Eh) of solution incubated with soil were measured over a time course. A soil sample was prepared by mixing 2.0 g of raw soil (andosol) and 0.02 g of wheat bran, followed by wrapping the mixture in nylon net with mesh size of 50 μm. The ratio of soil to wheat bran in the mixture corresponds to that of soil mixed with 1 t/10 a of wheat bran to the depth of 0 to 20 cm. As a control, soil without wheat bran was used. The soil was collected immediately before the experiment from the root zones of tomato plants in a greenhouse. The soil sample contained about 120 second-stage juveniles (J2s) of the root-knot nematode, *Meloidogyne incognita*, per 2 g soil. Because soil temperature should be about 30°C or higher for actual RSD, soil temperature was set at 30°C for the following experiments.

For DO measurement, an aqueous-phase Clark-type oxygen electrode with a cuvette (Rank Brothers Ltd., Cambridge) was used. The soil sample prepared as described above was placed in the electrode cuvette with 7 ml distilled water, which was tightly closed with a plunger. The DO in the resulting solution was continuously measured at 30°C for 24 hr while stirring with a magnetic stirrer. Before the measurement, air was passed through the distilled water in a conical flask at 30°C for three minutes to ensure that the distilled water was fully saturated with oxygen at atmospheric pressure.

For Eh measurement, the soil sample prepared as described above was placed in a test tube (2.5 cm in diameter) with 7 ml of distilled water; the electrode of an Eh/pH meter (D-21, HORIBA, Ltd., Kyoto) was placed in the solution and the test tube was tightly closed. The test tube was incubated in a 30°C water bath and the Eh of the solution was measured over a time course of 2 to 24 hr. Eh values were corrected to standard hydrogen electrode readings.

The soil sample was taken out after the incubation to isolate live J2s from it by the Baermann funnel technique, and number of recovered J2s was counted. After pH of the solution incubated with the soil sample was measured using an Eh/pH meter, VFAs in the solution were analyzed as described below.

Measurement of VFAs generation in a laboratory experiment:

Volatile fatty acids in soil solution were analyzed over a time course. A mixture of 53 g of air-dried soil (andosol) and 1.0 g of wheat bran was placed in eight bottles (100 ml), to which 65 ml of distilled water was added. The ratio of soil to wheat bran in the mixture corresponds to that of soil mixed with 1 t/10a of wheat bran to the depth of 0 to 20 cm. The bottles were thoroughly de-aerated, tightly closed with a stopper, and incubated in a 30°C water bath.

Before and 24, 48 and 72 hr after the incubation, soil was sampled from two bottles on each occasion. Eh and pH of soil-water mixtures (1:2.5 soil/water ratio) were measured with an Eh/pH meter. The soil sample (20 g) and distilled water (30 ml) were mixed and shaken on an orbital shaker for 15 min, centrifuged for 3 min in a micro-centrifuge, and the sediment was extracted once more with distilled water (20 ml) in the same way. Following gravity filtration of the sum of supernatant using a filter paper (No. 6, Toyo Roshi Kaisha, Ltd. Tokyo), VFAs in the supernatant were analyzed using a gas chromatography (GC-17A, Shimadzu Corp., Kyoto) equipped with a flame ionization detector and an HP-INOWAX column (length, 15 m; inner diameter, 0.53 mm; Hewlett-Packard, Inc., Palo Alto, CA). Molar concentrations of VFAs in the water-phase of the soil were calculated from soil moisture content.

Survival rate of J2s exposed to VFAs:

Meloidogyne incognita was allowed to parasitize tomato plants (cv. House Momotaro, Takii & Co., Ltd., Kyoto) grown in a greenhouse. Immediately before the experiment, J2s were isolated from greenhouse soil by the Baermann funnel technique and dispersed in distilled water to obtain about 150 J2s/ml.

Acetic acid and n-butyric acid were evaluated for nematicidal effect on J2s in accordance with Mojés method modified by Sano and Gotoh (1972). In a test tube of 2.5 cm in diameter and 11.5 cm in length, about 150 J2s were added to 30 ml of a VFAs aqueous solution with pH adjusted with aqueous solutions of sodium hydroxide and hydrochloric acid. The J2s were incubated at 30°C for 24 hr; for a control, about 150 J2s were added to 30 ml of distilled water. After incubation, J2s settled to the bottom of the test tube were taken out with a Komagome pipette and transferred to 30 ml of distilled water in another test tube of the same shape, which was allowed to stand still at room temperature for 4 hr and J2s settled to the bottom of the test tube were taken out with a Komagome pipette. This procedure was repeated twice to wash J2s with distilled water. Some treated J2s

stored in the distilled water at 25°C for 16 hr recovered from anesthetization. The J2s were transferred onto a filter paper (Kimwiper S-2000, Nippon Paper Crecia Co. Ltd., Tokyo) in a test tube with distilled water to count live J2s that moved downward and passed through the filter paper. The J2s count was taken as the number of surviving J2s. Because J2s were also lost in the control during the operation, a value of 100 was assigned to the survival rate of the control to correct the survival rate of the test. This calculation resulted in the survival rate of the test exceeding 100% in some cases.

Comparative nematicidal effects of VFAs and an analysis of their interactions:

Nematicidal effects of acetic acid and n-butyric acid were compared and their interactions were analyzed. J2s were immersed in aqueous solutions of 2.0, 4.0, 6.0, and 8.0 mM of acetic acid and n-butyric acid all adjusted to pH 4.5 or in mixtures of the two solutions for 24 hr to determine surviving J2s as described above. The assay was done in triplicates.

The combined VFAs interaction was evaluated by a nonlinear regression analysis using the equation (Haga and Sugiyama, 2007) given below:

$$y = \frac{100}{1+Exp(A+B(x_a+Cx_b+D\sqrt{x_a x_b}))}$$

where y = survival rate (%), x_a = concentration of acetic acid (mM), x_b = concentration of n-butyric acid (mM), *Exp* = exponential function, A = constant, B = constant, C = constant, and D = constant. C is the ratio of x_a effect to x_b effect. A value of 1 for C means that the two are equal in effect. D represents the interaction of x_a and x_b effects. A negative value of D means an antagonistic effect; zero means an additive effect; and a positive value means a synergistic effect. Data were statistically analyzed with JMP version 6.0.2 (SAS Institute Inc., Cary, NC).

Influence of pH on nematicidal effects of VFAs:

Influence of pH on nematicidal effects of acetic acid and n-butyric acid was evaluated. J2s were immersed in 7.5 mM aqueous solutions of acetic acid, n-butyric acid and citric acid (control), which were adjusted to pH 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0, for 24 hr to count surviving J2s by the method of Sano and Gotoh (1972) as described above. The experiment was done in triplicates.

Furthermore, J2s were immersed in the following acetic acid solutions for 24 hr to count surviving J2s: 4.0, 6.0, 8.0, 10, 12, and 14 mM solutions adjusted to pH 4.0; 6.0, 8.0, 10, 12, 14, and 16 mM solutions adjusted to pH 4.5; 10, 20, 30, and 40 mM solutions adjusted to pH 5.0; 20, 40, 60, and 80 mM solutions adjusted to pH 5.5; and 50, 100, 150, and 200 mM solutions adjusted to pH 6.0. Concentrations of nonionized acetic acid in the solutions were calculated using the Henderson-Hasselbalch equation (Hasselbalch, 1916) given below:

$$a = \frac{A}{\left[1+\frac{10^{(-pKa)}}{10^{(-pH)}}\right]}$$

where a = concentration of nonionized acetic acid in solution (mM), A = concentration of ionized and nonionized acetic acid in solution (mM), and pKa = 4.7 (equilibrium constant of acetic acid at 30°C). The experiment was done in triplicates. Logistic LC_{50} values were calculated with JMP version 6.0.2.

Method for the inoculation of potted tomato plants with J2s:

To confirm the results of the method of Sano and Gotoh (1972), whether J2s exposed to VFAs were alive was evaluated by the inoculation of potted tomato plants with the J2s and the result was compared with that of the Sano and Gotoh method.

A tomato plant (cv. Kyouryoku-beiju, Takii & Co., Ltd., Kyoto) at the age of two months was planted per pot (inside dimension: 24 cm, height: 30 cm) and grown in a greenhouse for about a month. Twenty potted plants were used for the inoculation test.

J2s, about 170, were immersed in 30 ml each of the following acetic acid solutions in a test tube for 24 hr at 30°C: 2.0 mM and 15 mM solutions adjusted to pH 4.5, 10 mM solutions adjusted to pH 3.0 and 6.5, and distilled water (pH close to 6.5). In each plot, J2s in seven test tubes were treated. After treatments, the number of surviving J2s in three test tubes was counted by the Sano and Gotoh method as described above. On the other hand, J2s in the other four test tubes were washed with 30 ml of distilled water as described above, which were injected to soil near the roots of four tomato plants respectively on June 5, 2004. The inoculated tomato plants were grown in a greenhouse until August 3 and the roots were indexed for root galls using a scale of 0 to 2, with 0 = no galls, 1 = some small galls, and 2 = numerous small galls.

Reductive soil disinfestation demonstration in a greenhouse:

As had been reported by Kubo *et al.* (2004), a field demonstration for RSD was conducted in a greenhouse (andosol) in Inzai City of Chiba Prefecture in June 2000. In the greenhouse, where melon had been grown from January to May and tomato had been grown from July to December every year, *M. incognita* had occurred throughout the year.

This greenhouse was divided into two plots: an RSD plot (108 m^2) and a control plot (54 m^2). Wheat bran (Chiba

Flour Milling Co., Ltd., Chiba), 2 t/10 a, was evenly spread over the surface of the RSD plot and thoroughly mixed with the soil to a depth of 0 to 40 cm by rotary tilling three times. In both plots, irrigation tubes (24 L $hr^{-1}m^{-1}$) were placed at intervals of about 80 cm with holes facing downward and transparent polyethylene film was placed over the irrigation tubes to cover the whole soil surface. The plots were irrigated for a half day and temporarily flooded. All openings of the greenhouse were closed to keep soil temperature high for 24 days from June 3 to 26. Then, the openings were opened, the film was removed, and the soil was returned to an oxidized state by rotary tilling.

A thermometer (Thermic model 2100A, Eto Denki Co., Tokyo) was used to measure soil temperature. The sensors were installed in the middle of each plot at depths of 20 and 40 cm during the demonstration period. Before and three days after the beginning of RSD on June 2, soil was sampled at the depths of 20 and 40 cm. Soil pH and Eh were measured as described above in a laboratory experiment.

Tomato plants (cv. Super Sun Cherry, Tokita Seed Co., Ltd., Saitama) were transplanted in the greenhouse on July 18 (2.2 plants/m^2). Soil was sampled at depths of 0 to 20 and 20 to 40 cm three and four months after planting to count the numbers of live J2s using the Baermann funnel technique. Before RSD, numbers of J2s per 20 g soil were 7.8 at the depth of 0 to 20 cm, and 18.5 at the depth of 20 to 40 cm. Four months after planting, the roots were washed free of soil and indexed for root galls using a scale of 0 to 10 (Bridge and Page, 1980), with 0 = no galling and 10 = 100% of roots galled, plant dead or dying.

RESULTS

Time-dependent changes in DO and Eh in the laboratory experiment:

When wheat bran was added to soil, DO in the solution sharply decreased as soon as the incubation began and reached 0 mM in 1.6 hr (Fig. 1A). When wheat bran was not added, DO also fell sharply to reach 0 mM in 5.0 hr. When wheat bran was added, Eh was about 450 mV at the beginning of the incubation, started to decrease 3 hr later, and reached -300 mV in 15 hr, indicating a quick change to a reduced state (Fig. 1B). In contrast, when wheat bran was not added, Eh did not decrease much and remained at 268 mV even 24 hr later. When wheat bran was added, Eh showed a complex pattern of changes after 24 hr, such as an increase up to 100 mV followed by a decrease (data not shown).

The experiment was repeated for different incubation times to measure Eh and VFAs concentrations in the solution incubated with the soil and count the number of live J2s in the soil sample at the end of incubation (Table 1). When wheat bran was not added, Eh and the number of J2s did not decrease and VFAs were below the detection limits. In contrast, when wheat bran was added, Eh and the number of J2s markedly decreased and acetic and n-butyric acids were detected. Formic, propionic, iso-butyric, n-valeric, and iso-valeric acids were below the detection limits whether wheat bran was added or not. Thus, RSD was confirmed to produce soil with a reduced state and generate acetic and n-butyric acid in the soil, followed by J2s death in the soil at 30°C in a short time.

Volatile fatty acids generation in the laboratory experiment:

Volatile fatty acids were not detected at the start of incubation (Fig. 2). When wheat bran was added, 2.6 mM of acetic acid was calculated in the water-phase in the soil after 24 hr of incubation, and 9.8 mM of acetic acid and 10.8

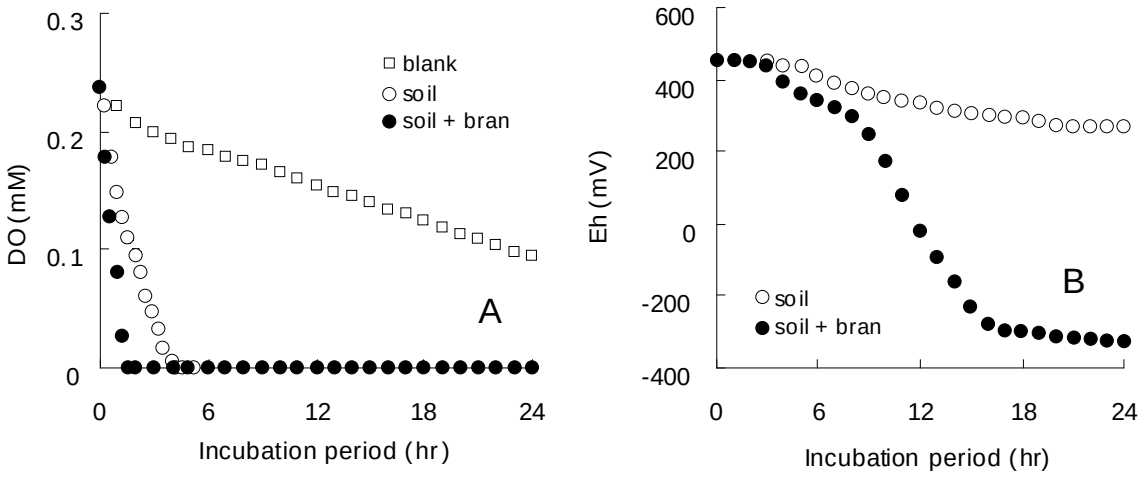

Fig. 1. Dissolved oxygen (DO) concentration (A) and redox potential (Eh) (B) of solution contained untreated soil and soil amended with wheat bran in a laboratory experiment. A blank test was performed to measure DO in solution incubated without a soil sample.

mM of n-butyric acid were calculated after 48 hr. Eh of the soil solution decreased to 135 mV after 24 hours of incubation and to -33 mV after 48 hr, generating soil with a reduced state.

In contrast, only an extremely small amount, 1.1 mM, of propionic acid was detected after 48 hr of incubation. Formic, propionic, iso-butyric, n-valeric, and iso-valeric acids were below the detection limits. All the VFAs were also below the detection limits in the control plot.

Comparison of acetic acid and n-butyric acid for nematicidal effect:

Acetic acid and n-butyric acid were compared for nematicidal effect because both acids were detected in the laboratory experiment. J2s were immersed in mixed solutions of 2.0 to 8.0 mM acetic acid and n-butyric acid adjusted to pH 4.5 for 24 hr. Both acids showed clear nematicidal effects at 4.0 mM with a decrease in the number of surviving J2s with increasing concentrations (Table 2). The number of surviving J2s also decreased in mixed solutions as in single-acid solutions.

Data in Table 2 were analyzed with a nonlinear regression to find how the survival rate was related to the concentration of aqueous solutions of both acids (Table 3). Parameter *C*, which compares effects of the two acids, was 1.14, revealing that the two acids were nearly equal in nematicidal effect. Parameter *D*, which shows interaction, was 0.08 with a 95% CI of -0.16 to 0.38. The data revealed that a mixture of the two acids showed an additive effect.

Influence of pH on the nematicidal effects of acetic acid and n-butyric acid:

To investigate the influence of pH on the nematicidal effects of VFAs, 10 mM acetic acid and n-butyric acid were adjusted to pH 3.0 to 6.5 to compare nematicidal effects of the pH-adjusted acids on J2s (Fig. 3). The survival rate of J2s exposed to the VFAs for 24 hr was nearly 0% at pH 3.0 and 4.0. The survival rate sharply increased at pH above 4.5 to reach about 100% at pH 5.5, where the two acids lost nematicidal activity almost completely. Acetic acid and n-butyric acid showed no difference in the relation between pH and nematicidal effect. In contrast, citric acid solutions used as controls showed no nematicidal effect in a pH range of 3.0 to 6.5.

To examine the influence of pH on the nematicidal effect of acetic acid in further detail, acetic acid solutions in a concentration range of 4.0 to 200 mM and a pH range of 4.0 to 6.0 were compared for nematicidal effect. Survival rates of J2s immersed in the aqueous solutions were plotted against concentrations of nonionized and ionized acetic acid (Fig. 4A). The survival rate decreased with increasing concentrations and decreasing pH. The survival rate was near-

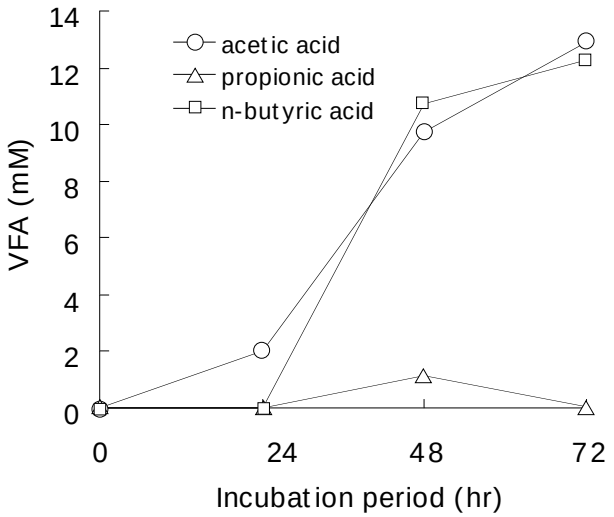

Fig. 2. Concentration of volatile fatty acids (VFA) in the water-phase of soil amended with wheat bran in a laboratory experiment. Values are the means of duplicates.

Table 1. Redox potential (Eh) and concentration of volatile fatty acids (VFAs) in solution incubated with soil samples, and the number of live second-stage juveniles (J2s) of *Meloidogyne incognita* recovered from the soil samples in a laboratory experiment

Sample	Incubation period (hr)	pH	Eh (mV)	No. of J2s per 2 g soil	VFAs (mM)	
					Acetic acid	n-Butyric acid
Soil	2	6.5	352	98	ND[1]	ND
	2	6.3	325	106	ND	ND
	9	6.4	280	80	ND	ND
	9	6.4	285	84	ND	ND
Soil + bran	2	6.5	383	83	0.35	ND
	5	6.1	101	29	ND	ND
	6	5.8	−264	0	1.51	0.37
	7	6.1	7	3	0.33	ND
	8	5.9	−243	6	1.10	0.24

[1] ND: not detected (< 0.1 mM).

Table 2. Number of surviving second-stage juveniles of *Meloidogyne incognita* affected with mixing ratios of acetic acid and n-butyric acid at pH 4.5 in a laboratory experiment.

Acetic acid (mM)	n-Butyric acid (mM)				
	0	2.0	4.0	6.0	8.0
0	133.7 ± 9.9[1]	107.0 ± 14.1	56.3 ± 11.3	12.7 ± 4.3	0.7 ± 0.7
2.0	103.7 ± 9.2	63.0 ± 4.9	14.3 ± 0.3	0.3 ± 0.3	
4.0	84.7 ± 13.5	7.3 ± 2.3	0.7 ± 0.3		
6.0	11.7 ± 3.2	2.0 ± 1.0			
8.0	5.3 ± 1.2				

[1] Values are the means±SEM of triplicates.

Table 3. Parameter estimates and 95% confidence intervals obtained from a nonlinear regression analysis of the relation between the survival rate of *Meloidogyne incognita* and mixing ratios of acetic acid and n-butyric acid as shown in Table 2.

Parameter	Estimate	Approximate SEM	95% confidence interval	
			Lower	Upper
A	−3.70	0.41	−4.91	−2.87
B	0.89	0.10	0.70	1.16
C	1.14	0.07	0.99	1.31
D	0.08	0.12	−0.16	0.38

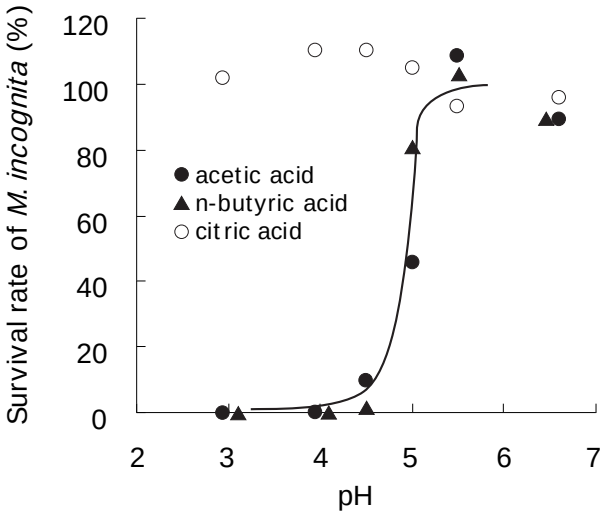

Fig. 3. Relation between the survival rate of *Meloidogyne incognita* and pH of acetic, n-butyric and citric acid at a concentration of 10 mM. Values are the means of triplicates.

ly 0% in the following combinations of pH and acetic acid concentration: pH 4.0 and 14 mM, pH 4.5 and 16 mM, pH 5.0 and 40 mM, pH 5.5 and 80 mM, and pH 6.0 and 200 mM. Survival rates were then plotted against the nonionized acetic acid concentrations calculated from the pH values (Fig. 4B). The relations between concentration of nonionized acetic acid and survival rate were nearly identical for all the pH values and the logistic LC_{50} value was estimated to be 5.6 ± 0.2 (SEM) mM.

Evaluation of the nematicidal effect of acetic acid using potted tomato plants:

Effects of RSD on J2s were evaluated by using the Sano and Gotoh method and the inoculation test with potted tomato plants. When J2s were immersed in a 15 mM acetic acid solution adjusted to pH 4.5 and a 10 mM acetic acid solution adjusted to pH 3.0, the number of live J2s was zero and no galls were generated in the inoculated tomato plants (Table 4). In the other plots, J2s survived and galls were generated in the inoculated tomato plants.

Reductive soil disinfestation demonstration in a greenhouse:

The soil temperature reached 35.0°C at the depth of 20 cm in the RSD plot on day 3 of the treatment, which was above the standard temperature of 30°C for RSD (Table 5). Eh in the soil solution decreased to -207 mV at the depth of 20 cm and -98 mV at the depth of 40 cm, revealing that the soil was in an intensely reduced state on day 3 of the treatment. In addition, 5.7 mM of acetic acid and 1.5 mM of n-butyric acid were calculated in the water-phase of the soil at the depth of 20 cm in the RSD plot. In the control plot, soil temperature reached 28.8°C at the depth of 20 cm, but Eh

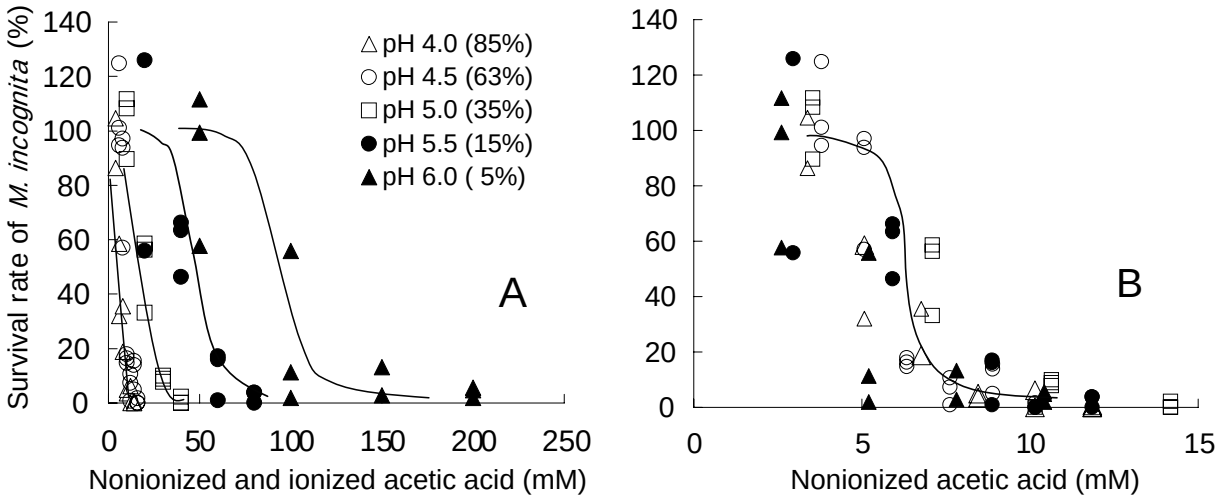

Fig. 4. Relation between the survival rate of *Meloidogyne incognita* and acetic acid. (A) Survival rate affected by acetic acid concentrations (4.0 to 200 mM) and pH (4.0 to 6.0). (B) Survival rate affected by nonionized acetic acid concentration calculated from the pH values. Symbols are the same in A and B. Numbers in parentheses are the percentage of nonionized acetic acid in nonionized and ionized acetic acid. Values are the means of triplicates.

Table 4. Number of surviving second-stage juveniles (J2s) of *Meloidogyne incognita* exposed to acetic acid solutions and root galling index of tomato plants inoculated with nematodes after exposure.

Acetic acid solution		No. of J2s[1]	Root galling index[2]
pH	Concentration (mM)		
6.5	0	118 ± 6	2, 2, 2, 2
4.5	2	116 ± 15	2, 2, 1, 1
4.5	15	0	0, 0, 0, 0
6.5	10	92 ± 8	2, 2, 1, 1
3.0	10	0	0, 0, 0, 0

[1] No. of surviving J2s counted by the method of Sano and Gotoh (1972). Values are the means±SEM of triplicates.
[2] Galling index: 0 = no galls; 1 = some small galls; 2 = numerous small galls. Four tomato plants in each plot were indexed.

Table 6. Effects of reductive soil disinfestation (RSD) on the density of second-stage juveniles (J2s) of *Meloidogyne incognita* and galling of tomato roots in a field demonstration.

Treatment	Soil depth (cm)	No. of J2s per 20 g soil[1]		Root galling index[2]
		3 months	4 months	
RSD	0－20	8	48	2
	20－40	15	214	
Control	0－20	1644	237	8
	20－40	534	765	

[1] No. of J2s counted three and four months after planting. Values are the means of four soil samples. No. of J2s before RSD was 8 per 20 g soil at the depth of 0-20 cm, and 19 at the depth of 20-40 cm.
[2] Galling index: 0 = no galling; 10 = 100% of roots galled, plant dead or dying (Bridge and Page, 1980). Values in RSD and control plots are the medians of 20 and 12 tomato plants respectively.

Table 5. Soil temperature, redox potential (Eh), and concentration of volatile fatty acids (VFAs) in control soil and soil amended with wheat bran for reductive soil disinfestation (RSD) in a field demonstration.

Treatment	Time of measurement[1]	Depth (cm)	Temperature (℃)	Eh[2] (mV)	pH[2]	VFAs[2](mM)	
						Acetic acid	n-Butyric acid
RSD	Before treatment	20	25.3	515	6.8	ND[3]	ND
		40	25.7	558	6.6	ND	ND
	3 days after treatment	20	35.0	－207	6.4	5.7	1.5
		40	22.7	－98	6.5	2.5	0.4
Control	Before treatment	20	27.5	511	6.8	ND	ND
		40	26.5	546	6.6	ND	ND
	3 days after treatment	20	28.8	511	7.0	ND	ND
		40	25.5	518	7.0	ND	ND

[1] Before treatment: June 2, 2000, 9:00; 3 days after treatment: June 5, 2000, 16:00.
[2] Values are the means of two soil samples.
[3] ND: not detected (< 0.1 mM).

did not drop markedly and VFAs were below the detection limit. Formic, propionic, iso-butyric, n-valeric, and iso-valeric acids were below the detection limits in both plots.

The soil temperature increased gradually and the highest temperatures at the depths of 20 and 40 cm in the RSD plot and the control plot were 42.0, 32.0, 37.2, and 30.0°C respectively on day 6 to 7 of the treatment. In Funabashi City near Inzai City where this demonstration was performed, the mean air temperature was 21.8°C during this demonstration; the lowest air temperature was 14.8°C; the highest air temperature was 30.0°C; and the total period of sunshine was 81.4 hr (AMeDAS, Japan Meteorological Agency).

The population density of J2s was much lower in the RSD plot than in the control plot both at the depths of 0 to 20 cm and 20 to 40 cm three months after planting (Table 6). Root galling index was also much lower in the RSD plot than in the control plot.

DISCUSSION

Reductive soil disinfestation was shown to be very effective for controlling nematodes in the laboratory experiment (Table 1). The RSD plot and the control plot were under the same temperature conditions and surmised to be under the same anaerobic conditions (Fig. 1A). In contrast, Eh decreased to under -200 mV only in the RSD plot (Fig. 1B). Ten mM levels of acetic acid and n-butyric acid were calculated in the water-phase of the soil under reductive conditions with an Eh -33 mV (Fig. 2) and had nearly equal nematicidal activity (Fig. 3), and their mixtures showed an additive effect (Tables 2 and 3). In the field demonstration, 5.7 mM of acetic acid and 1.5 mM of n-butyric acid were calculated at the depth of 20 cm, although the soil pH was not low enough to form nonionized acids at lethal concentra-

tions, and RSD was confirmed to be effective for controlling nematodes (Tables 5 and 6). Therefore, VFAs generation is suggested to be a factor in the mechanism of RSD in practice. Momma *et al.* (2006) also showed that VFAs are factors contributing to suppression of soil-borne pathogens by RSD.

Volatile fatty acids have long been known for their nematicidal effects (Stephenson, 1945; Johnston, 1959; Banage and Visser, 1965; Nagase *et al*., 1982; Bansal and Bajaj, 2003; Browning *et al*., 2004; McElderry *et al*., 2005) and noted as a group of naturally occurring chemicals during decomposition (Sayre *et al*., 1965; Hollis and Rodriguez-Kabana, 1966; Elmiligy and Norton, 1973; Lynch, 1978; Badra *et al*., 1979; Djian *et al*., 1991; McBride *et al*., 2000; Chitwood, 2002). Differences in nematicidal activity were found among VFAs (Stephenson, 1945; Johnston, 1959; Badra *et al*., 1979; Bansal and Bajaj, 2003; McElderry *et al*., 2005), and that of acetic acid was lowest (Nagase *et al*., 1982). On the other hand, VFAs were reported to have same nematicidal effects (Banage and Visser, 1965). The LC_{50} values of acetic and n-butyric acids for *Rotylenchuslus reniformis* were reported to be 135 and 270 ppm (2.1 and 3.1 mM) respectively; and 120 and 64 ppm (1.9 and 0.7 mM) on *Tylenchulus semipenetrans*, respectively (Badra *et al*., 1979). The result of this study obtained by the Sano and Gotoh method and statistical data analysis revealed that acetic acid and n-butyric acid had no pronounced difference in nematicidal effect and their interaction was additive (Fig. 3, Tables 2 and 3). The LC_{50} value of nonionized acetic acid for *M. incognita* was estimated to be 5.5 mM (Fig. 4B). The result obtained by the Sano and Gotoh method was confirmed from the result of the inoculation test (Table 4). Therefore, the sum of molar concentrations of acetic acid and n-butyric acid is believed to be a possible indicator for RSD effects.

In addition, pH of aqueous solution is known to exert intense influence on the nematicidal effect of VFAs (Stephenson, 1945; Sayre *et al*., 1965; Banage and Visser, 1965; Hollis and Rodriguez-Kabana, 1966; Elmiligy and Norton, 1973; Djian *et al*., 1991; McElderry *et al*., 2005). We also showed that pH greatly influenced the nematicidal effect of VFAs (Table 4 and Fig. 3). From a lack of nematicidal activity by citric acid in a pH range of 3.0 to 6.5, the nematicidal effect of VFAs was inferred to result from their intrinsic property, and not the pH of solution surrounding nematodes. Also, an analysis of pH on the nematicidal effect of acetic acid revealed that the nematicidal effect depended on the amount of nonionized acetic acid (Fig. 4). VFAs are dissociated in an aqueous solution and the degree of dissociation depends on its dissociation constant (pKa) and the pH of the solution. This suggests that more acetic acid molecules are dissociated with increasing pH to lower the nematicidal effect of the acid.

In this study, pH was confirmed to decrease to 5.8 in RSD-treated soil but pH values lower than 5.5 have not been observed. The laboratory experiments suggest that nematicidal effects of VFAs generated by RSD are extremely low at such pH. However, the pH in microsites surrounding wheat bran particles and nematodes may be more important in elucidating the mechanisms of RSD than the overall soil pH. Soil is a complex mixture of mineral, organic, gaseous, and aqueous phases and its properties vary at microsites of soil aggregates (Norton, 1978). The pH of nitrification microsites was considerably lower than the pH measured in the bulk soil (Strong *et al*., 1997). Hence, VFA generation is surmised to cause localized decreases in pH around wheat bran particles mixed into soil and exert sufficient nematicidal effect on nematodes present at these localized sites with low pH.

It is necessary to consider ammonia (Nagase *et al*., 1982; Culbreath *et al*., 1986) and nitrous acid (Forge, *et al*., 2005) as other nematicidal substances generated under reductive conditions. Ammonium nitrogen is generated in RSD-treated soil (Ushio *et al*., 2004) and increases pH gradually. Therefore, it may be that ammonia decreases the nematicidal effect of VFAs over a time course. Further studies are necessary on the nematicidal substances in RSD as well as on changes in soil environments including pH.

Lack of oxygen may also be considered a condition lethal to nematodes. In this experiment, VFAs generation resulting from decreased Eh, not lack of oxygen, was considered an important factor in the mechanism of RSD. On the other hand, in an RSD experiment involving *Pratylenchus penetrans*, the nematicidal effect due to lack of oxygen was pronounced when soil temperature rose higher than 30°C (Katase *et al*., 2005). Therefore, soil temperature and lack of oxygen seem to be important nematicidal factors in a shallow layer of RSD-treated soil in a greenhouse, where the effect of solar heat is pronounced enough to increase soil temperature above 30°C. The intrinsic RSD effect conceivably appears in soil layers below this shallow layer.

In general, soil microorganisms are believed to deplete oxygen and then generate VFAs under anaerobic conditions. Hence, RSD may be considered as a cultural control taking advantage of functions of soil microorganisms. Labile organic matter, such as molasses (Shinmura, 2003) and alcohol (Uematsu *et al*., 2008), have also been tried for RSD. RSD is expected to replace synthetic agricultural chemicals in greenhouse cultivation with favorable condi-

tions for this method.

ACKNOWLEDGEMENT

We thank Dr. T. Haga (formerly Tokyo University of Science) for his advice on a nonlinear regression analysis. This work was partly supported by the Ministry of Agriculture, Forestry and Fisheries as a part of the research project “Development of new biorational techniques for sustainable agriculture”.

LITERATURE CITED

Badra, T., Saleh, M. A. and Oteifa, B. A. (1979) Nematicidal activity and composition of some organic fertilizers and amendments. Revue de Nématologie 2, 29-36.

Banage, W. B. and Visser, S. A. (1965) The effect of some fatty acids and pH on a soil nematode. Nematologica 11, 255-262.

Bansal, R. K. and Bajaj, A. (2003) Effect of volatile fatty acids on embryogenesis and hatching of *Meloidogyne incognita* eggs. Nematologia Mediterranea 31, 135-140.

Bridge, J. and Page, S. L. (1980) Estimation of root-knot nematode infestation levels on roots using a rating chart. Tropical Pest Management 26, 296-298.

Browning, M., Dawson, C., Alm, S. R. Gorres, J. H. and Amador, J. A. (2004) Differential effects of butyric acid on nematodes from four trophic groups. Applied Soil Ecology 27, 47-54.

Chitwood, D. J. (2002) Phytochemical based strategies for nematode control. Annual Review of Phytopathology 40, 221-249.

Culbreath, A. K., Rodriguez-Kabana, R. and Morgan, J. G. (1986) Chitin and *Paecilomyces lilacinus* for control of *Meloidogyne arenaria.* Nematropica 16, 153-166.

Djian, C., Pijarowski, L., Ponchet, M., Arpin, N. and Favre-Bonvin, J. (1991) Acetic acid: a selective nematicidal metabolite from culture filtrates of *Paecilomyces lilacinus* (Thom) Samcon and *Trichoderma longibrachiatum* Rifai. Nematologica 37, 101-112.

Elmiligy, I. A. and Norton, D. C. (1973) Survival and reproduction of some nematodes as affected by muck and organic acids. Journal of Nematology 5, 50-53.

Forge, T. A., Bittman, S. and Kowalenko, C. G. (2005) Impacts of sustained use of dairy manure slurry and fertilizers on populations of *Pratylenchus penetrans* under tall fescue. Journal of Nematology 37, 207-213.

Haga, T. and Sugiyama, K. (2007) Applicaton of a nonlinear regression analysis with JMP (II). SAS Institute Japan Ltd., 99pp. (in Japanese)

Hasselbalch, K. A. (1916). Die berechnung der wasserstoffzahl der Blutes aus der frien gebundenen kahlensaure desselben, und die Sauerstoffbindung des Blutes als funktion der wasserstoffzahl. Biochemische Zeitschrift 78, 112-144.

Hollis, J. P. and Rodriguez-Kabana, R. (1966) Rapid kill of nematodes in flooded soil. Phytopathology 56, 1015-1019.

Johnston, T. M. (1959) Effect of fatty acid mixtures on the rice stylet nematode (*Tylenchorhynchus martini* Fielding, 1956). Nature 183, 1392.

Katase, M., Watanabe, H., Sito, T., Kubota, S. and Kadono, F. (2005) Effects of disinfestation by soil reduction on root-lesion nematodes and its application to nematode control in strawberry fields. Bulletin of the Chiba Prefectural Agriculture Research Center 4, 117-123. (in Japanese with English summary)

Kubo C. and Katase, M. (2007) Effect and promotion of disinfestation by soil reduction on soil-borne diseases and nematode. Plant Protection 61, 68-72. (in Japanese)

Kubo, C., Katase, M., Shimizu, K., Kato, H. and Takeuchi, T. (2004) Effect of disinfestation by soil reduction on soil-borne diseases and nematode. Bulletin of the Chiba Prefectural Agriculture Research Center 3, 95-104. (in Japanese with English summary)

Lynch, J. M. (1978) Production and phytotoxicity of acetic acid in anaerobic soils containing plant residues. Soil Biology and Biochemistry 10, 131-135.

McBride, R. G., Mikkelsen, R. L. and Barker, K. R. (2000) The role of low molecular weight organic acids from decomposing rye in inhibiting root-knot nematode populations in soil. Applied Soil Ecology 15, 243-251.

McElderry, C. F., Browing, M. and Amador, J. A. (2005) Effect of short-chain fatty acids and soil atmosphere on *Tylenchorhynchus* spp. Journal of Nematology 37, 71-77.

Momma, N., Yamamoto, K., Simandi, P. and Shishido, M. (2006) Role of organic acids in the mechanisms of biological soil disinfestations (BSD). Journal of General Plant Pathology 72, 247-252.

Nagase, A., Kuwahara, Y., Tominaga, Y. and Sugawara, R. (1982) Nematicidal activity of akylamine against the pine wood nematode, *Bursaphelenchus lignicolus*. Agricultural and Biological Chemistry 46, 167-172.

Nakasono, K. (1989) Effects of barnyard manure amendments on the growth of tomato plants inoculated with *Meloidogyne incognita*. Japanese Journal of Nematology 19, 38-45. (in Japanese with English summary)

Norton, D. C. (1978) Ecology of Plant-parasitic Nematodes, John Wiley & Sons, Inc., New York, 268pp.

Sano, Z. and Gotoh, A. (1972) Application of the Mojé method for testing the susceptibility of nematodes to

chemicals. Kyushu Plant Protection Research 18, 1-6. (in Japanese with English summary)

Sayre, R. M., Patrick, Z. A. and Thorpe, H. J. (1965) Identification of a selective nematicidal component in extracts of plant residues decomposing into soil. Nematologica 11, 263-268.

Shinmura, A. (2003) Control effect to subsoil of soil sterilization by soil reduction method to use theriac. Annals of the Phytopathological Society of Japan 69, 78. (abstract in Japanese)

Shinmura, A., Sakamoto, N. and Abe H. (1999) Control of Fusarium root rot of Welsh onion by soil reduction. Annals of the Phytopathological Society of Japan 65, 352-353. (abstract in Japanese)

Sikora, R. A. (1992) Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. Annual Review of Phytopathology 30, 245-270.

Sotomayor, D., Allen, Jr., L. H., Chen, Z., Dickson, D. W. and Hewlett, T. (1999) Anaerobic soil management practices and solarization for nematode control in Florida. Nematropica 29, 153-170.

Stephenson, W. (1945) The effects of acids on a soil nematode. Parasitology 36, 158-164.

Strong, D. T., Sale, P. W. G. and Helyar, K. R (1997) Initial soil pH affects the pH at which nitrification ceases due to self-induced acidification of microbial microsites. Australian Journal of Soil Research 35, 565-570.

Uematsu. S., Tanaka-Miwa, C., Sato, R., Kobara, Y. and Sato, M. (2008) Ethyl alcohol treatment as a promising material of reductive soil disinfestation for controlling root-knot nematode and soil-borne diseases. Japanese Journal of Phytopathology 74, 46. (abstract in Japanese)

Ushio, S., Katase, M., Kubo, C., Yamamoto, F., Ootsuka, E. and Anzai, T. (2004) Changes of chemical properties of protected field andosol during disinfestation by soil reduction. Bulletin of the Chiba Prefectural Agriculture Research Center 3, 105-112. (in Japanese with English summary)

Received February 25, 2009